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Acclimated green microalgae consortium to treat sewage in an alternative urban WWTP in a coastal area of Central Italy

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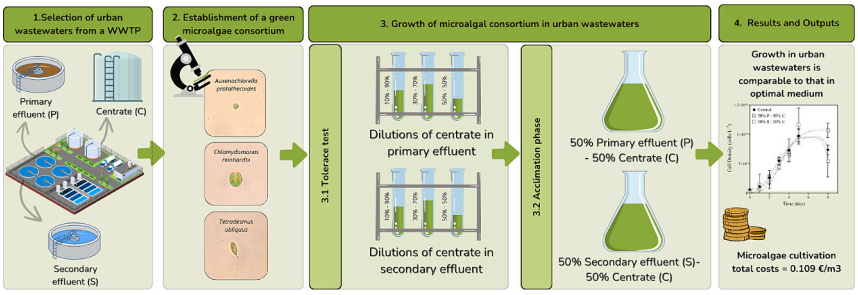
HIGHLIGHTS

- Microalgae consortium acclimated to different combinations of wastewater streams.
- No clear difference was seen using primary or secondary effluents with 50 % centrate.
- Alteration of photosynthetic activity was detected in wastewater-grown microalgae.
- Nutrient removal was close to 100 % but CO_2 should be added to avoid high pH values.
- The microalgae system integrated to WWTP was assessed to cost 0.109 €·m^{-3}.

GRAPHICAL ABSTRACT



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ABSTRACT

This study exposed a microalgal consortium formed by *Auxenochlorella protothecoides*, *Tetradesmus obliquus,* and *Chlamydomonas reinhardtii* to six mixed wastewater media containing different proportions of primary (P) or secondary (S) effluents diluted in centrate (C). Algae could grow at centrate concentrations up to 50 %, showing no significant differences between effluents. After acclimation, microalgae cultivated in 50%P-50%C and 50%S-50%C grew at a rate similar to that of control cultures (0.59–0.66 d^{-1}). These results suggest that the consortium acclimated to both sewage streams by modulating the proportion of the species and their metabolism. Acclimation also altered the photosynthetic activity of wastewater-grown samples compared to the control, probably due to partial photoinhibition, changes in consortium composition, and changes in metabolic activity. No major differences were observed between the two streams with respect to biochemical composition, biomass yield, or bioremediation capacity of the cultivated algae but algae grown in the secondary effluent showed qualitatively higher exopolysaccharides (EPS) production than algae grown in primary. Regarding wastewater remediation, microalgae grown in both WW media showed proficient nutrient removal efficiencies (close to 100 %); however, the final pH value (close to 11) would be controversial if the system were upscaled as it is over the legal limit and would cause phosphorus precipitation, so that CO_2 addition would be required. The theoretical scale-up of the

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microalgae system could achieve water treatment costs of 0.109 €·m^{-3}, which was significantly lower than the costs of typical activated sludge systems.

1. Introduction

Although current sewage treatment plants can perform well in terms of removing macro-pollutants, they are highly demanding in terms of energy and chemical reagents and emit significant amounts of greenhouse gases (Foglia et al., 2023; Pikaar et al., 2022; Marinelli et al., 2021). The modern circular economy approach is boosting innovative technologies to make the wastewater (WW) treatment sector carbon-neutral, as required in future European legislation (COM, 2024), promote resource recovery, and guarantee safety standards (Foglia et al., 2023). In this respect, microalgal cultivation systems appear to be sustainable and low-carbon alternatives for treating different WW streams, including urban WW (Lens and Khandelwal, 2023; Satya et al., 2023). Microalgal systems can be integrated with typical activated sludge treatment processes to decrease the relative impacts of conventional WW treatment plants (WWTPs) (Mantovani et al., 2020; Nishshanka et al., 2023). The treatment scheme of WWTPs varies depending on the sewage stream to be treated, that is, the primary and secondary effluents or the supernatant of the anaerobic digestates (known as the centrate) (Fig. S1). Microalgae can remediate WW streams and can fix CO_2 and produce biomass that can be used to obtain multiple bio-products, such as biofertilisers or biostimulants (Amaya-Santos et al., 2022; González-Camejo et al., 2021; Cao et al., 2023; Cunha-Chiamolera et al., 2024). Therefore, microalgae-based systems promote circular economic principles in the water sector and offer economic opportunities that must be evaluated in detail.

Despite the potential benefits, microalgae-based treatment technologies face challenges and knowledge gaps persist (Araújo et al., 2021; COM, 2022a). From a technical perspective, the limited treatment efficiency and lack of robustness of microalgal cultivation systems are significant barriers to overcome (Lens and Khandelwal, 2023). The limited activity and robustness of microalgae cultures are related to the high variability of climatic conditions in the long-term operation of microalgae systems (Morillas-España et al., 2021), as well as to the presence (or absence) of certain compounds that can limit microalgae growth, such as salinity (Yang et al., 2022; Zhang et al., 2023), which can be relevant in coastal areas affected by seawater intrusion (Foglia et al., 2020).

Primary effluents contain significant amounts of solids and bacteria, whereas secondary effluents are often nutrient-limited (Belachqer-El Attar et al., 2023; Nishshanka et al., 2023). Centrates can contain significant amounts of solids and potentially toxic compounds such as ammonia and sulphide, which have been reported to limit microalgae activity even at low concentrations (González-Camejo et al., 2017; Rossi et al., 2020). Mixing sewage streams can reduce these limitations and improve microalgal performance and culture resilience (Al-Mallahi and Ishii, 2022; Gao et al., 2023). As the specific characteristics of sewage streams and their combinations can compromise the design of large-scale systems, it is important to investigate this issue. Green microalgae of the genera *Chlorella, Chlamydomonas,* and *Tetradesmus* are commonly used to treat WW because of their high resilience and rapid growth capacity (Arias et al., 2019; Pachés et al., 2020, Mohsenpour et al., 2021, Li et al., 2019, Su, 2021). These microalgae can implement mixotrophic metabolism, that is, they can use both autotrophic and heterotrophic metabolism depending on substrate availability (Ferreira et al., 2019); however, they tend to favour photoautotrophic metabolism under appropriate lighting conditions (Babaei et al., 2016). The ability to use organic C as an energy source also reduces the chemical oxygen demand (COD) in WW streams. Although the cultivation of single microalgal species in WW has been investigated by many authors (Gao et al., 2023; Wu et al., 2023), microalgal consortia are usually preferable to monocultures because they generally present higher resilience owing to the functional diversity and metabolic exchange that contribute to a steady algal density (Gururani et al., 2022; Mandal and Corcoran, 2022; Sahu et al., 2023; Mollo et al., 2024). Nevertheless, insights into the acclimation capacity of the consortium to medium and outdoor conditions, as well as on the resilience against physical, chemical, or biological factors, such as photoinhibition and the presence of inhibitors or microbial competitors, remain poorly understood. A better understanding of these issues would be of great interest for the optimal control of the microalgae process as it can help assess the possible drivers that would produce variations in the photosynthetic performance of the microalgae consortium, allowing them to be quantified so they can be detected early.

This preliminary study evaluated the capacity of a green microalgal consortium to treat different combinations of mixed sewage streams, assessing its ability to acclimate and maintain efficient bioremediation and biomass production. The evaluation was performed considering a multidisciplinary approach that interconnects plant physiology and environmental engineering knowledge with the aim of providing relevant information for the future implementation of a microalgae cultivation unit to be integrated with conventional WW treatment systems. The results are particularly relevant for the application of microalgal cultivation technologies in coastal areas where saline intrusion is common and will provide relevant information for selecting the most suitable WW treatment scenario and mixing ratios between primary or secondary effluents and centrates, as well as for setting the initial conditions for outdoor microalgae cultivation at a demonstrative scale.

2. Material and methods

2.1. Experimental design

A consortium of three green algae species was established by co-inoculating *Auxenochlorella protothecoides* (CCAP 211/8D, https://www.ccap.ac.uk/), *Tetradesmus obliquus* (CCAP 276/3A), and *Chlamydomonas reinhardtii* (RCC125, https://roscoff-culture-collection.org/) cultures. It was then propagated for at least ten generations. A similar consortium was used to grow synthetic digestate and showed good growth performance and remediation (Mollo et al., 2024). Algal consortium cultures were placed in tubes filled with 30-mL synthetic freshwater medium BG11 (Pandey et al., 2023). Tubes were maintained at room temperature (23 ± 3 °C) and illuminated with continuous (24 h·d^{-1}) cool white, fluorescent lamps (300 μmol photons m^{-2} s^{-1} at the tubes' surface).

WW samples were obtained from the Falconara-Marittima WWTP (Ancona, Italy), specifically from primary effluent (P), secondary effluent (S), and centrifuged digestate, the so-called centrate (C).

For the first set of the experiments (tolerance test), a fixed amount of initial microalgal cell concentration (10^5 cells·mL^{-1}) was transferred from standard medium BG11 to tubes containing 30 mL of 6 combinations of WW streams to assess the most suitable scenario for the upscaled microalgae reactor, i.e., a combination of primary effluent and centrate (Fig. S1a) or combination of secondary effluent and centrate (Fig. S1b), the following media were tested: i) 10%P-90%C; ii) 30%P-90%C; iii) 50%P-50%C; iv) 10%S-90%C; v) 30%S-90%C; vi) 50%S-50%C (Table 1). Three independent biological replicates were established and compared to consortium cultures grown in BG-11, selected as the control condition (Lee et al., 2023). This synthetic medium provides a balanced quantity of nutrients for optimal growth under laboratory phototrophic conditions, thus highlighting the degree of change in algal metabolism/physiology and alterations in microalgae performance caused by WW

composition. The cultures were manually shaken at least twice daily for proper gas exchange inside the tubes.

The WW streams which yielded the best growth performance were then selected for the second set of experiments, which were 50%P-50%C and 50%S-50%C. For this second step, the algal consortium was acclimated for >10 generations to these selected WW media. At least three biological replicates of acclimated cells were established in 500-mL Erlenmeyer flasks filled with 300-mL WW streams and compared to control cultures grown in BG11. The initial microalgal concentration was fixed at 10^5 cells·mL^{-1}. The cultures were manually shaken at least twice daily for proper gas exchange inside the flasks. Biochemical composition, photosynthetic efficiency, and consortium species abundance were assessed in acclimated cells harvested during the exponential phase (day 4 of the growth curve).

2.2. Growth analysis and dry weight

Growth was assessed by measuring the cell number daily using a CASY TT cell counter (Innovatis AG, Reutlingen, Germany), as described previously (Petrucciani et al., 2022). The same technique was used to determine the mean volume of cells present in the consortium.

A non-linear regression (β-function, Yin et al., 2003) on the daily measured cell density carried out on a minimum of three distinct cultures for each treatment was used to obtain the specific maximum growth rates (μ_{max}). The model of β-function Eq. (1) has been already applied to algal growth (Mollo et al., 2023; Petrucciani et al., 2023). The C_m parameter was used to derive μ_{max} using Eq. (2), where Nt_m is the density of cells achieved at time t_m (inflection point at which growth rate is maximum).

$$\frac{dN}{dt} = C_m\left(\frac{t_e - t}{t_e - t_m}\right)\left(\frac{t - t_b}{t_m - t_b}\right)^{\frac{t_m - t_b}{t_e - t_m}} \quad (1)$$

$$\mu_{max} = \frac{C_m}{Nt_m} \quad (2)$$

Dry weight was attained by washing the algal culture twice in MQ water (to remove salts) and drying the biomass at 80 °C in pre-weighted tubes; measurements were carried out on samples taken from three different replicates during the exponential and stationary phases.

2.3. Consortium species composition

Imaging flow cytometer (IFC) FlowSight® (Amnis Corp., Seattle, WA) with the INSPIRE software package (Amnis Corp.) was exploited to evaluate both species composition in the consortium and cellular morphological characteristics. Cells collected during the exponential growth phase were analysed following protocol and settings already described by Petrucciani et al. (2023). The IDEAS software package was used for post-acquisition data analysis. Only cells showing chloroplast autofluorescence, as determined by the intensity feature of the IDEAS software, were included in the cell metrics. Among the single-well-focused living cells, larger cells (*C. reinhardtii* and *A protothecoides*) were gated and separated from smaller cells (*T. obliquus* and *A. protothecoides*) to assess whether a shift in cell volume composition occurred in the consortium (Fig. S2). To obtain detailed population variations of the three species, cells were separated and gated according to their different areas and aspect ratios (details of the analysis are shown in Fig. S3). Height, width, area, circularity, diameter, and perimeter (quantification of cell circumference; IDEAS User Manual, version 6.0, March 2013) were the morphological features used in the analysis. The numbers given as outputs and used for statistical analysis represent the average values of the cited features calculated for at least 10,000 cells for each biological replica.

2.4. Pigment quantification and photosynthetic analysis

Algal cultures were sampled during the exponential growth phase and centrifuged at 1500 *g* for 8 min; then, 2 mL 100 % (*v*/v) methanol was added to pellets. Samples were stored overnight at −20 °C to completely extract pigments (Ritchie, 2006). Supernatants were separated from the pellet by centrifugation (13,000*g* for 5 min) and their absorbance was measured spectrophotometrically (UV-1900i, SHIMADZU CORP). Absorbance (Abs) at 664, 647, and 470 nm was used to quantify chlorophyll *a* and b using Eqs. (3a) and (3b) described by Ritchie (2006) and chlorophytes and carotenoids using Eq. (4) described by Wellburn (1994).

$$\text{Chlorophyll } a\ (\mu g\ mL^{-1}) = 11.8668 \cdot Abs_{664nm} - 1.7858 \cdot Abs_{647nm} \quad (3a)$$

$$\text{Chlorophyll } b\ (\mu g\ mL^{-1}) = 18.9775 \cdot Abs_{647nm} - 4.8950 \cdot Abs_{664nm} \quad (3b)$$

$$\text{Carotenoids}\ (\mu g\ mL^{-1}) = (1000Abs_{470nm} - 1.63Chl\,a - 104.96Chl\,b)/221 \quad (4)$$

Samples collected during the exponential phase were used to assess the in vivo variable fluorescence of photosystem II (PSII) chlorophyll *a* using a dual-pulse amplitude modulation (PAM) 100 fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Microalgal cells were harvested by centrifugation (1500 ×*g*, 5 min), resuspended in 2 mL fresh growth medium at a concentration of $3{\cdot}10^6$ cells mL^{-1}, and acclimated in the dark for 30 min. The samples were then transferred to glass cuvettes and analysed under continuous stirring.

Table 1
Characterisation of sewage streams used to obtain the cultivation media.

	10%P-90%C	30%P-90%C	50%P-50%C	10%S-90%C	30%S-70%C	50%S-50%C	Control
pH	7.5 ± 0.1	7.5 ± 0.0	7.5 ± 0.1	7.0 ± 0.2	7.1 ± 0.1	7.2 ± 0.0	7.6
Conductivity (μS·cm^{-1})	4536 ± 52	4608 ± 42	4680 ± 41	8073 ± 69	7359 ± 74	6645 ± 63	–
COD (mgCOD·L^{-1})	295 ± 6	537 ± 9	779 ± 20	198 ± 45	462 ± 63	725 ± 77	–
TSS (mg·L^{-1})	92 ± 21	167 ± 42	242 ± 11	66 ± 17	147 ± 31	228 ± 25	N.D.
Alkalinity (mgCaCO$_3$·L^{-1})	366 ± 35	472 ± 65	579 ± 33	241 ± 25	375 ± 54	509 ± 47	–
N-NH$_4$ (mgN·L^{-1})	28.0 ± 1.2	47.3 ± 2.0	66.7 ± 0.3	11.6 ± 2.2	34.6 ± 3.4	57.6 ± 2.7	N.D.
N-NO$_2$ (mgN·L^{-1})	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.2	N.D.
N-NO$_3$ (mgN·L^{-1})	1.9 ± 0.1	2.7 ± 0.3	3.5 ± 0.1	9.9 ± 1.6	8.9 ± 0.9	7.9 ± 0.3	247.1
N$_{inorg}$ (mgN·L^{-1})	30.6 ± 1.5	50.7 ± 2.2	70.8 ± 0.7	21.6 ± 1.8	43.7 ± 0.9	65.8 ± 2.8	247.1
P-PO$_4$ (mgP·L^{-1})	3.8 ± 0.2	8.4 ± 0.1	13.0 ± 0.4	3.6 ± 0.1	8.3 ± 0.5	12.9 ± 1.1	7.1
P$_{tot}$ (mgP·L^{-1})	5.3 ± 0.2	10.9 ± 0.1	16.5 ± 0.4	4.9 ± 0.2	10.5 ± 0.6	16.2 ± 1.3	7.1
Cl$^-$ (mgCl·L^{-1})	1332 ± 5	1239 ± 23	1145 ± 78	2959 ± 13	2504 ± 55	2049 ± 124	951
SO_4^{2-} (mgSO_4^{2-} ·L^{-1})	259 ± 10	240 ± 20	221 ± 11	488 ± 58	418 ± 33	349 ± 22	122
Na$^+$ (mgNa·L^{-1})	774 ± 8	746 ± 44	719 ± 9	1711 ± 29	1475 ± 68	1239 ± 49	472
K$^+$ (mgK·L^{-1})	43 ± 5	44 ± 4	45 ± 4	87 ± 7	78 ± 11	69 ± 4	18
Mg$^+$ (mgMg ·L^{-1})	171 ± 9	164 ± 10	158 ± 6	341 ± 23	297 ± 25	253 ± 8	12
Ca^{2+} (mgCa·L^{-1})	331 ± 6	374 ± 28	417 ± 3	404 ± 12	431 ± 21	458 ± 37	10

ND: Non detected.

Photosynthetic parameters were obtained from the light curve protocol preceded using a pre-treatment as described by Hemme et al. (2014): after determination of the maximum efficiency of photosystem II (Fv/Fm), cells were illuminated for 5 min with far-red light followed by 5 min of dark acclimation. A new Fv/Fm value was determined before starting the measurements and is presented in Table 3. The measuring light was set at 5 μmol photons·m^{-2}·s^{-1} and saturation pulse was fixed at 6000 μmol· photons·m^{-2}·s^{-1} for 600 ms. For light curve kinetics cells were stepwise exposed to increasing light intensities (from 6 to about 2000 μmol photons·m^{-2}·s^{-1}) every 1 min for a total of 20 steps. Fluorescence-derived parameters, that is Fv/Fm, non-photochemical quenching (NPQ), and electron transfer rate (ETR(II)), were calculated as Eqs. (5)–(7) (Baker, 2008):

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \tag{5}$$

where Fv/Fm is the maximum efficiency of photosystem II, Fm is the maximal fluorescence of dark-adapted cells, and Fo is the minimal fluorescence of dark-adapted cells.

$$NPQ = \frac{F_m - F_{m'}}{F_{m'}} \tag{6}$$

where NPQ is the non-photochemical quenching and Fm' is the maximal fluorescence from light-adapted cells (Baker, 2008).

$$ETR\ (II) = PAR \times A \times \text{fraction PSII} \times \varphi PSII \tag{7}$$

where ETR is the electron transport rate through photosystem II, PAR is the light intensity applied, A is the fraction of incident light absorbed (usually assumed to be 0.84), fraction PSII is the fraction of PSII over total PS (usually assumed to be 0.5), and φPSII = (Fm'-F)/Fm'.

Light curves ETR(II) data were fitted according to the model of Platt et al. (1980) and the parameters were calculated using Dual-PAM software: (i) maximum electron transport rate *ETRmax* [μmol electrons ·m^{-2}·s^{-1}], (ii) *Ik* is the light intensity at which light saturation sets in [μmol photons ·m^{-2}·s^{-1}], and (iii) α the initial slope of the light response curve, which is related to the quantum efficiency of photosynthesis [electrons/photons].

2.5. Elemental and macromolecular composition

Exponentially growing cells were collected to determine their organic and inorganic compositions. The cells were harvested by centrifugation (1500 ×*g* for 8 min) and washed twice with Milli-Q water. Pellets were used for total protein quantification according to the Lowry method described by Peterson (1977) and detailed by Petrucciani et al. (2022). A UV-1900i spectrophotometer (SHIMADZU Corp.) was used to measure absorbance at 750 nm which was interpolated into a standard curve constructed using known concentrations of bovine serum albumin to quantify the total amount of protein in the samples.

Aliquots of washed cell suspensions were dried at 80 °C on a silicon window for FTIR spectroscopy (Tensor 27 FTIR spectrometer, Bruker Optics, Ettlingen, Germany). The spectra of the whole algal cells were acquired following the protocol detailed in a previous study (Domenighini and Giordano, 2009). The relative abundances of cellular pools (lipids, proteins, and carbohydrates) were calculated via band integrals of deconvolved spectra after band assignment, as explained by Giordano (2001) (OPUS 6.5 software was used, Bruker Optics GmbH, Ettlingen, Germany). Semi-quantification of carbohydrates and lipids was achieved by comparing the total measured protein content with the FTIR absorbance ratio between the pool of interest and the proteins, according to Palmucci et al. (2011).

Qualitative analysis of exopolysaccharides (EPS) production was carried out following the procedure detailed by Crayton (1982). Cells were stained in vivo with a solution of 0.1 % *w/v* alcian blue 8GX in 0.5 N acetic acid (total EPS) or 0.1 % w/v alcian blue in 0.5 N HCl (sulphated EPS).

For the elemental composition, aliquots of washed cells suspension were dried at 80 °C and analysed using an elemental analyser (ECS 4010, Costech Italy) connected to the ID Micro EA isotope ratio mass spectrometer (Compact Science Systems, LymedaleBusiness Centre, Newcastle-under-Lyme, United Kingdom) to assess both C and N quotas and their stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$), following the protocol detailed in Petrucciani et al. (2022). Elements other than C and N were quantified in the cell biomass using a total reflection X-ray fluorescence spectrometer (S2 Picofox; Bruker AXS Microanalysis GmbH, Berlin, Germany), following the procedure described by Petrucciani et al. (2022). Spectral deconvolution and quantification of elemental abundances were performed using the SPECTRA 6.1 software (Bruker AXS Microanalysis GmbH, Berlin, Germany).

2.6. WW remediation

The bioremediation capacity of both acclimated cultures (50%P-50% C and 50%S-50%C) was evaluated by calculating the removal efficiency during the cultivation period, i.e., the difference between the concentration of the pollutants contained in the WW at the beginning of the test (Table S1) and at the end (when maximum microalgae concentration was expected). The main objective was to compare the two scenarios. The pollutants evaluated were COD, macronutrients (N and P), and main ions. The COD was measured following Standard Methods (APHA, 2012), whereas the main nutrient species (NH_4, NO_2, NO_3, and PO_4) and other ions (Na, K, Ca, Mg, Cl, and SO_4) were analysed using inductively coupled plasma mass spectrometry (Dionex ICS-1000 and 1100).

2.7. Statistical analysis

To assess the significant differences among the means of the dependent variables (i.e., growth rates, elemental contents, and macromolecular composition) in the different WW streams and the control condition (independent variables), a one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test was used. GraphPad Prism 8.0.2.263 (GraphPad Software, San Diego, CA, USA) was used to perform the statistical tests, and the level of significance was set at 0.05.

Average values of the morphological features described in Section 2.3 were analysed using principal component analysis (PCA) using PAST 4.03 (Paleontological Statistics Software Package for Education and Data Analysis) (Hammer et al., 2001). *Z*-values ((n-mean)/SD) were used to normalise the input values.

2.8. Economic assessment

The microalgae-based process evaluated at the laboratory scale was theoretically upscaled for integration in the Falconara Marittima WWTP (Ancona, Italy) (Fig. S4). A flow diagram of the treatment process is shown in Fig. 1. The cultivation technology selected was high-rate algal ponds (HRAPs) coupled with ultrafiltration membranes for the separation of microalgal biomass from water, similar to that reported by Robles et al. (2020).

The reactor volume was designed to treat all the centrate generated by the Falconara Marittima WWTP (348 m^3·d^{-1}). This corresponds to 50 % of the total influent, which was mixed with 50 % of the primary effluent of the WWTP. This implies a total treatment flow of 696 m^3·d^{-1}. Primary effluent was selected as this would imply a reduction in the amount of WW to be fed to the activated sludge reactor, unlike the secondary effluent. Considering the growth rate obtained under laboratory conditions (Section 3.2), the hydraulic retention time (HRT) of the system was assumed as 3.5 d (slightly longer than the theoretical optimum HRT of 3.3 d). Consequently, the HRAP total volume accounted for 2434 m^3, corresponding to an area of 0.8 Ha, considering a 20-cm deep reactor (Rossi et al., 2024).

The total annual cost (TAC) of the microalgae system was calculated

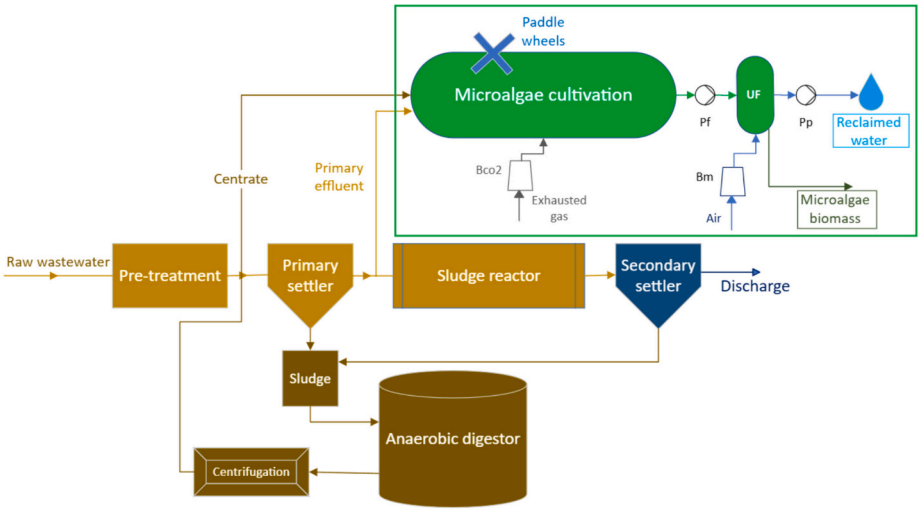

Fig. 1. Flow diagram of the upscale microalgae cultivation system integrated into Falconara Marittima WWTP.

including capital (CAPEX) and operating expenses (OPEX) using Eq. (8), which was reported by Ferrer et al. (2015):

$$TAC = \frac{r\,(1+r)^t}{(1+r)^t - 1} \cdot CAPEX + OPEX \quad (8)$$

where TAC is the total annual cost ($€\cdot y^{-1}$), *r* is the annual discount rate (5 % as in Jiménez-Benítez et al., 2024), *t* is the depreciation period in years (20 years for civil infrastructure and 10 years for equipment, Jiménez-Benítez et al., 2024), CAPEX is the total capital expenditures (€), and OPEX is the yearly operational expenditure ($€\cdot y^{-1}$).

With respect to the CAPEX, the price of the HRAP, land acquisition, paddle wheels of the reactors, concentrated (Pc) and permeate (Pp) pumps, blowers for CO_2 supply (Bco_2), membrane scouring (Bm) (Fig. 1), and fees were considered. Complementary equipment and infrastructure were considered as additional costs (Table S2). The remaining equipment and infrastructure were not included as they already existed in the WWTP. Similarly, only the OPEX of the microalgae system was calculated, that is, power requirements (both energy and power tariffs), chemical reagents for membrane cleaning, membrane and equipment replacement, civil works, and building maintenance. Personnel costs were not included because no staff increase was expected to operate the microalgal system integrated with the current WWTP. Details of the assumptions considered for both CAPEX and OPEX as well as the economic assessment methodology can be found in Section S1.

3. Results and discussion

3.1. Tolerance test and selection of WW medium

Growth rates (μ) showed no significant differences among the sewage combinations (Fig. 2), with values ranging from 0.19 ± 0.02 to 0.22 ± 0.03 d^{-1} (*p*-values >0.05, Table S3) but were significantly lower than under control conditions, at 0.43 ± 0.09 d^{-1}. These growth rate values were lower than those reported for other green microalgae grown in sewage (0.4–0.9 d^{-1}; Arbib et al., 2017; Pachés et al., 2020). A possible explanation is the high salinity and conductivity in the WW streams tested, which were significantly higher than normal values for sewage (Table 1). However, salinity has been reported to limit freshwater microalgae activity at concentrations much higher than those measured in this study, i.e., in the range of 10–20 $gNaCl\cdot L^{-1}$ (Sahle-Demessie et al., 2019; Yang et al., 2022; Zhang et al., 2023). Further evaluation is needed as this issue could be relevant for the replicability of microalgae cultivation technology in coastal areas that are affected by seawater intrusion (such as the Mediterranean region).

Unlike the growth rates (Table S3), the final microalgal cell

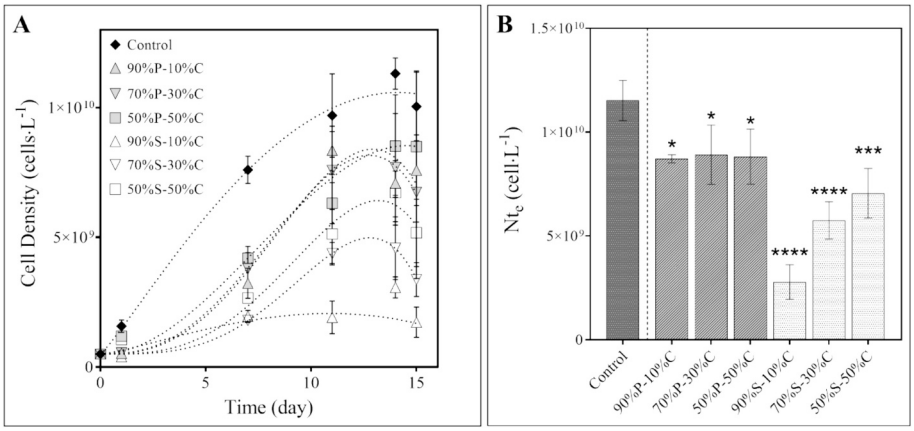

Fig. 2. Growth analysis of algal consortium grown in different combinations of streams. A) Growth curves with experimental data expressed as mean ± SD and dashed lines representing regression curves. Different symbols and colours indicate different experimental conditions. B) Algal density achieved at stationary phase. Nt_e calculated in different experimental conditions; data are expressed as mean ± SD. Asterisks indicate significant differences between each experimental condition and control condition (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

concentrations varied according to the growth in the sewage media. Specifically, algae grown in media containing primary effluent achieved significantly higher cell densities than those grown in media containing secondary effluent (Fig. 2B). One reason could be related to nutrient limitations. The low amounts of N, P, and inorganic C (indirectly measured as alkalinity) in the secondary effluent compared to those in the primary effluent and centrate (Table 1) could have limited the final number of microalgal cells. Znad et al. (2018) reported a similar trend using sewage from an Australian WWTP, obtaining higher growth of *Chlorella vulgaris* when grown in primary effluent than in secondary effluent. Moreover, the higher salinity of the secondary effluent, which was almost twice that of the primary effluent and centrate (Table 1), could affect algal growth.

The highest percentage of centrate (50 %) resulted in the same N_{te} achieved with the addition of 10 % centrate in the presence of primary effluent (Fig. 2). Previous studies focusing on centrate remediation by green microalgae reported that 5–30 % of centrate in growth media was the maximum range before microalgae photosynthetic activity was limited by the toxicity of ammonia, sulphide, or other compounds (Clagnan et al., 2022; Romero-Villegas et al., 2018). Consequently, the combination with the highest concentration of centrate (50%P-50%C and of 50%S-50%C) was considered the most suitable water medium for microalgal growth as it provided the highest amount of nutrients without limiting microalgal activity significantly. Using up to 50 % centrate in microalgae-based side-stream treatment systems could facilitate the operation of conventional activated sludge systems by decreasing the pollutant load recirculating into the mainstream environment (Mantovani et al., 2020).

3.2. Characterisation of the algal consortium acclimated to selected WW media

On the base of the tolerance tests, WW media containing 50%P-50%P and 50%S-50%C were selected as growth media for microalgae acclimation. Acclimation is the physiological response of cells to stressors that induce quantitative or /qualitative alterations in the expressed proteome (Giordano, 2013). The cells were acclimated for at least 10 generations and then characterised. Remarkably, acclimated cells showed similar growth to the control cultures in terms of growth rate, cell density, and biomass (Fig. 3; Table 2). Indeed, compared to tolerance tests (Fig. 2), cells which underwent the acclimation phase presented approximately 3-fold higher growth rates and much shorter lag phases, thus obtaining a growth performance similar to that of cells grown in optimal medium (Fig. 3, Table 2). Hence, the acclimated consortium is expected to be suitable for outdoor upscaling. The microalgal biomass concentration was similar for all the cultivation media (Table 2). These values were within the range of those reported by Dickinson et al. (2013) and Feng et al. (2020) under similar microalgae cultivation conditions but were lower than the values reported by Leong et al. (2020) and Znad et al. (2018) for *C. vulgaris* (Table S4). It must be noted that these values were obtained under laboratory-scale cultivation conditions (such as light irradiance and penetration, light photoperiod, temperature, mixing, and pCO_2) and the urban WW composition. Thus, the values obtained in this study are preliminary and could differ significantly from those under continuous outdoor conditions.

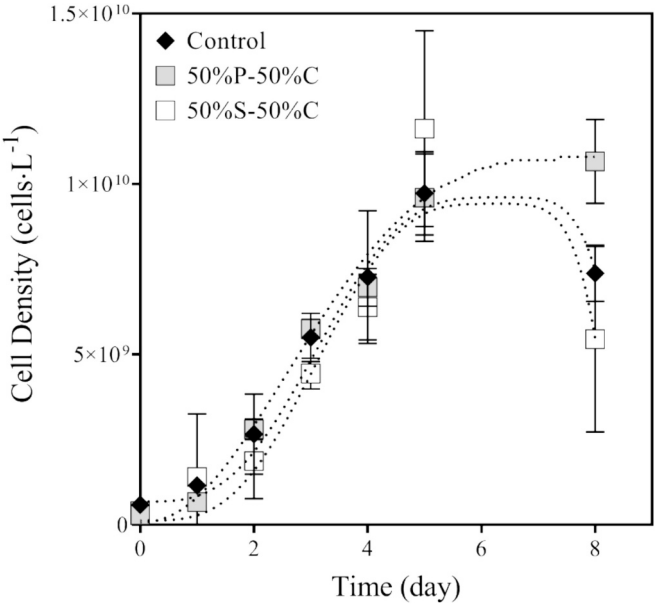

Fig. 3. Growth curves of algal consortium acclimated to the sewage combinations (50 % primary effluent (P)-50 % centrate (C); 50 % secondary effluent (*S*)-50%C). Experimental data are expressed as mean ± SD and dashed lines representing regression curves ($n > 3$). Different symbols and colours indicate different experimental conditions as detailed in the graph legend.

Table 2
Growth parameters and biomass characterisation of acclimated cells grown in the selected WW media; data are expressed as mean ± SD (n > 3). Different letters indicate significant differences among conditions of each parameter ($p < 0.05$).

	Control	50%P-50%C	50 % S-50%C
μmax (d^{-1})	0.59 ± 0.01[a]	0.60 ± 0.07[a]	0.66 ± 0.06[a]
Nt_e ($cells{\cdot}L^{-1}{\cdot}10^{10}$)	0.97 ± 0.07[a]	1.1 ± 0.1[a]	0.94 ± 0.02[a]
Biomass at day 4 ($g{\cdot}L^{-1}$)	0.35 ± 0.03[a]	0.30 ± 0.08[a]	0.24 ± 0.04[a]
Biomass at day 8 ($g{\cdot}L^{-1}$)	0.56 ± 0.05[a]	0.58 ± 0.03[a]	0.59 ± 0.02[a]
Proteins ($fg{\cdot}\mu m^{-3}$)	0.17 ± 0.02[a]	0.22 ± 0.05[a]	0.15 ± 0.03[a]
Lipids (arbitrary unit)	0.0042 ± 0.0018[a]	0.0030 ± 0.0013[ab]	0.0012 ± 0.0009[b]
Carbohydrates (arbitrary unit)	0.26 ± 0.08[a]	0.52 ± 0.21[a]	0.34 ± 0.04[a]
Chlorophyll *a* ($fg{\cdot}\mu m^{-3}$)	1.7 ± 0.2[a]	1.0 ± 0.1[b]	1.6 ± 0.1[a]
Chlorophyll *b* ($fg{\cdot}\mu m^{-3}$)	0.7 ± 0.2[a]	0.3 ± 0.1[b]	0.45 ± 0.02[ab]
Carotenoids ($fg{\cdot}\mu m^{-3}$)	0.51 ± 0.11[a]	0.38 ± 0.04[ab]	0.64 ± 0.05[b]

The overall biomass quality was assessed during the exponential phase (Table 2). Results revealed that the macromolecular composition of WW-acclimated microalgal cells in terms of proteins, lipids, and carbohydrates was comparable to that of cells grown in control medium, except for the pigment content, which was lower in cells acclimated to 50%P-50%C (Table 2). Therefore, nutrient supply by these centrate-rich media not only allowed the proper growth of microalgae but also the maintenance of fair macromolecular homeostasis. This can be relevant for the possible market expansion of microalgae-based bio-products as variability in the production of microalgae bio-products is a major challenge (Rossi et al., 2024; van Duinen, 2023); however, this should be confirmed during the continuous cultivation of microalgae.

Nevertheless, the algal consortium underwent relevant changes to acclimate to the WW media in terms of the relative abundance of the species (Figs. 4, S2). PCA analysis of morphological features showed that cells in control condition differed significantly from those in WW, according to PC1 (96.48 % of the total variance; Fig. 4A, Table S5). All measured parameters (height, width, area, circularity, diameter, and perimeter) contributed equally to the separation (Table S6). In particular, the percentage of larger *C. reinhardtii* cells decreased from approximately 45 % in the control condition to 10–20 % in the WW combinations (Fig. 4B). Indeed, the cells in the consortium had half the average volume when acclimated to WW conditions (Fig. 3C). This suggests that smaller cells were more competitive and thrived at the

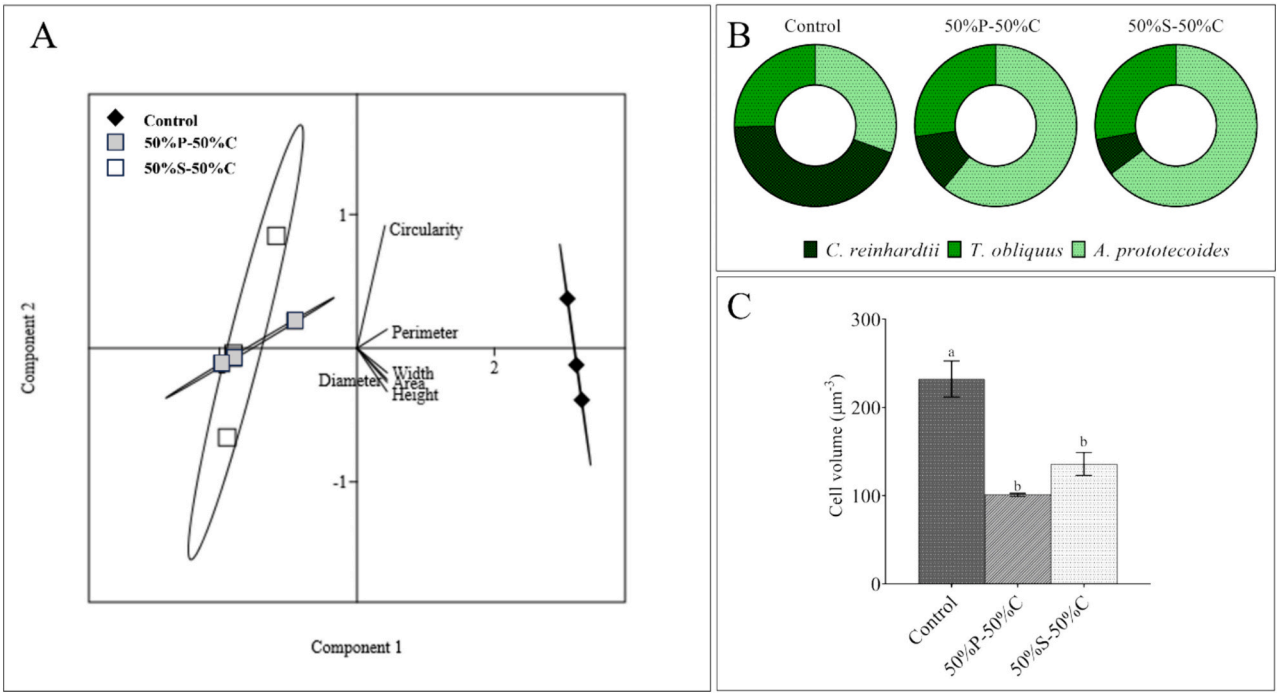

Fig. 4. Morphological characterisation of algal consortium acclimated to two different combinations of sewage streams compared to the control condition (50 % primary effluent (P)-50 % centrate (C); 50 % secondary effluent (*S*)-50%C). A) PCA analysis on algal morphological features (see Section 2.3). B) Percentage (%) of species in the consortium in the exponential growth phase. C) Average cellular volume of the algal consortium. Data are represented as mean ± SD. Letters represent significant differences among conditions ($p < 0.05$).

expense of larger cells under the unbalanced nutrient regime of WW. Moreover, the need for a greater S/V ratio to efficiently absorb inorganic and organic compounds by osmotrophy (Candido and Lombardi, 2020) may have favoured the shift of the algal consortium towards smaller species (Fig. 4). It cannot be ruled out that the change in relative species abundance could be related to acclimation to pollutants in the centre of both WW media, as reported by González-Camejo et al. (2017) who described a shift in the dominant species occurring in a microalgae consortium in response to the sulphide concentration.

Despite the good growth performance of the consortium acclimated to sewage streams (Fig. 3), a significant decrease in Fv/Fm was observed (Table 3) compared to the control condition. Furthermore, when the samples were illuminated with subsequent steps of increasing light intensities, that is, light curve experiments to measure fluorescence-based parameters, microalgae grown in WW medium displayed lower photosynthetic electron transport throughout all light curve tests (Fig. 5A), as estimated by ETR(II). In addition to ETRmax, the other parameters obtained by fitting the ETR(II) curve, namely, alfa and Ik, were lower in the WW-grown cultures than in the BG11 control (Table 3). This indicates that, in addition to reduced electron transport, WW-grown algae had lower light (quantum) use efficiency and photosynthesis was saturated at a lower light intensity than the control.

Despite minor differences in NPQ (parameter indicating the fraction of light energy dissipated as heat) between the control and WW-grown algae, the control values linearly increased with increasing light intensity unlike the WW samples, in which NPQ values reached roughly steady state values for light intensities over about 1200 μmol photons·m^{-2}·s^{-1}. This suggests that the WW-grown consortium is more susceptible to light-induced damage under strong illumination (Fig. 5B).

A combination of factors may have contributed to the altered photosynthetic activity in the WW-grown samples. First, the changes in species composition occurring in the consortium (Fig. 4) may have altered their overall photosynthetic responses, in line with the species-specific features of photosynthesis regulation (Goss and Lepetit, 2015; Lacour et al., 2020; Minagawa and Tokutsu, 2015). Furthermore, the lower light intensity required to saturate photosynthesis in the WW samples (Table 3, Fig. 5) suggests that they are more prone to over-reduction of the photosynthetic electron transport chain when exposed to light, a condition which may lead to oxidative damage to the photosynthetic apparatus and inactivation of photosystems (i.e., photoinhibition), reducing the overall photosynthetic capacity (Behera et al., 2019; Straka and Rittmann, 2018). This could be relevant to the upscaling technology. At higher scales, microalgae cultivation is affected by non-optimal outdoor illumination and the use of WW as a cultivation medium may exacerbate such suboptimal conditions. The design of a large-scale photobioreactor selected as a cultivation system, as well as its cultivation control systems, should consider these challenges (Barceló-Villalobos et al., 2019; Fierro et al., 2023; Nwoba et al., 2019). Because both WW showed similar results, a change in the WW stream used to cultivate microalgae was not expected to result in significantly different responses to variable light irradiance.

Table 3
Photosynthetic efficiency of algal consortium acclimated to two different combinations of sewage streams compared to the control condition (50 % primary effluent (P)-50 % centrate (C); 50 % secondary effluent (*S*)-50%C). Data are represented as mean ± SD. Letters represent significant differences among conditions ($p < 0.05$).

	Control	50%P-50%C	50 % S-50% C
Fv/Fm	0.75 ± 0.01^{a}	0.56 ± 0.04^{b}	0.58 ± 0.02^{b}
ETR max (μmol electrons·m^{-2}·s^{-1})	90 ± 7^{a}	32 ± 6^{b}	38 ± 6^{b}
α (el./ph)	0.37 ± 0.02^{a}	0.28 ± 0.06^{ab}	0.25 ± 0.02^{b}
Ik (μmol photons·m^{-2}·s^{-1})	240 ± 30^{a}	123 ± 44^{b}	154 ± 36^{ab}

Fv/Fm: Maximum quantum efficiency of PSII Photochemistry; *ETRmax:* maximum electron transport rate; *Ik:* light intensity at which light saturation sets; *α*: the initial slope of the light response curve, which is related to the quantum efficiency of photosynthesis [electrons/photons].

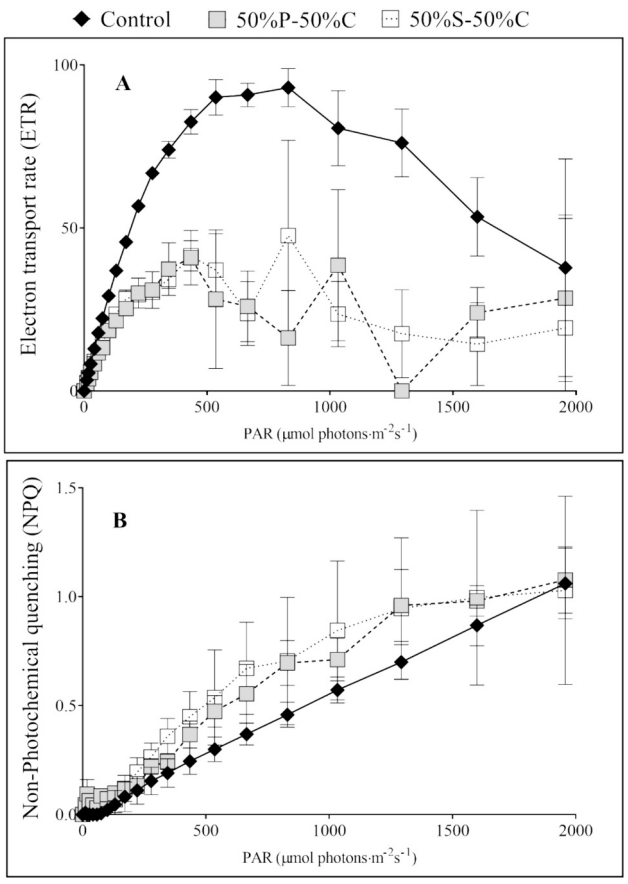

Fig. 5. Photosynthetic efficiency of algal consortium acclimated to two different combinations of sewage streams compared to the control condition (50 % primary effluent (P)-50 % centrate (C); 50 % secondary effluent (S)-50% C). A) Electron Transport rate (ETR). B) Non-photochemical quenching (NPQ). Data are represented as mean ± SD.

The significant presence of nitrogen in the form of ammonium (NH_4^+)/ammonia (NH_3) in WW may represent another challenge for photosynthesis in the microalgal consortium. When present in high amounts, ammonium ions can uncouple the membrane electrochemical potential, impairing all processes dependent on delta-pH formation across the photosynthetic membranes, such as ATP synthesis, or the activation of protection mechanisms, such as NPQ, thus indirectly impairing photosynthesis (Britto and Kronzucker, 2013; Coskun et al., 2017). In addition, ammonia can bind to and damage the oxygen-evolving complex of photosystem II and negatively affect the activity of photosystem I and dark respiration rates (Markou et al., 2016; Markou and Muylaert, 2016). In the WW combinations used in the present study, the total reduced N concentration (NH_4^+/NH_3) started from values of 46–47 mg·L^{-1} (Table S1); these values would be expected to be tolerated by most green algae (Chai et al., 2021; Gutierrez et al., 2016). However, due to the high pH values in these WW media at the end of the test (10.9 ± 0.2 and 10.8 ± 0.2, for 50%P-50%C and 50%S-50%C, respectively), a significant amount of total reduced N was assumed to be in the form of ammonia (NH_3), which is in acid-base equilibrium with ammonium, i.e., 25 ± 1 % and 19 ± 4 % for 50%P-50%C and 50%S-50%C, respectively. Rossi et al. (2020) reported that these amounts of ammonia could limit the photosynthetic activity of green microalgae by up to 20–25 %.

Another possible explanation for reduced photosynthesis may be the modulation of microalgal metabolism. The similar growth rate and biomass yield between the control and WW samples (Table 2) suggest that WW-grown microalgae were not significantly limited in their growth, despite the reduced photosynthetic electron transport rate. As organic matter was available in the WW (Table S1), algae could have implemented a mixotrophic metabolism and downregulated photosynthetic activity (Candido and Lombardi, 2020, 2018; Marchello et al., 2018). This hypothesis is consistent with the data obtained; however, independent of the aforementioned reasons for lower photosynthetic efficiency, the acclimatory response shown by the consortium grown in mixed effluents allowed the same C quota per cell volume reached by control cultures (Table 4). The N content per cell volume was similar in all samples. However, shifts in $\delta^{13}C$ and $\delta^{15}N$ were recorded (Fig. 6), confirming a modulation in the main metabolic processes. The change in C fractionation was due to a change in the C source used by the microalgae for assimilation. Different C sources are characterised by different C stable isotope signatures. This is true not only for a change in the main inorganic C source (i.e., CO_2 or bicarbonate) but also if organic C sources are exploited (Peterson and Fry, 1987). In addition, the activity of the enzymes involved in molecule uptake and processing inside the cell contribute to the detected $\delta^{13}C$. For example, during photosynthetic assimilation of CO_2, the enzyme Rubisco selectively discriminates and assimilates the lighter isotope (C^{12}) rather than the heavy one (C^{13}), causing an increased C fractionation in the biomass ($\delta^{13}C$ more negative). In contrast, in cells acclimated to WW, a decrease in C fractionation occurred compared to that in the BG11-grown culture (Fig. 6). This could indicate the major use of bicarbonate for CO_2 fixation (Petrucciani et al., 2022, 2023, 2024). However, urban WW used in this work contained limited amounts of carbonates and bicarbonates for optimal microalgae growth, with mass C:N ratios only accounting for 0.91 ± 0.25 and 0.79 ± 0.33 for 50%P-50%C and 50%S-50%C, respectively. These ratios were considerably low as the C:N typical ratio in microalgae biomass is 6.6 (C:N:P = 106:16:1) (Reynolds, 2006). Consequently, due to the availability of organic matter in the media, high surface tension of WW, (Yadav and Sen, 2017) and availability of organic matter, mixotrophic metabolism was favoured, allowing the assimilation of both inorganic and organic carbon contained in WW (Ferreira et al., 2019). In addition, N fractionation decreased, proving that assimilated N was enriched in the heavier isotope N^{15} which was derived from human disposal of WW, compared to assimilated N in the

Table 4
Amounts of elements per unit volume and stoichiometry in the algal consortium acclimated to two different combinations of sewage streams compared to the control condition (50 % primary effluent (P)-50 % centrate (C); 50 % secondary effluent (S)-50%C). Quotas are expressed as mean ± SD and C: N: P molar ratio. Letters indicate significant differences among the conditions ($p < 0.05$).

	Control	50%P-50%C	50 % S-50%C
C (fg·μm^{-3})	153 ± 20 ^{a}	206 ± 72^{a}	171 ± 36^{a}
N (fg·μm^{-3})	30 ± 5^{a}	37 ± 13^{a}	31 ± 8^{a}
P (fg·μm^{-3})	4.2 ± 0.5^{a}	14.5 ± 4.2^{b}	12.9 ± 0.5^{b}
Molar C: N:P	46 ± 6: 8 ± 1: 1 ± 0.2	18 ± 6: 3 ± 1: 1 ± 0.3	16 ± 3: 3 ± 1: 1 ± 0.03
Mass C: N:P	37 ± 5: 7 ± 1: 1 ± 0.1	14 ± 5: 3 ± 1: 1 ± 0.3	15 ± 6: 2 ± 1: 1 ± 0.04
S (fg·μm^{-3})	1.3 ± 0.2^{a}	1.6 ± 0.3^{a}	1.5 ± 0.3^{a}
K (fg·μm^{-3})	1.6 ± 0.3^{a}	3.0 ± 0.6^{b}	2.4 ± 0.2^{ab}
Ca (fg·μm^{-3})	0.4 ± 0.1^{a}	23.9 ± 6.2^{b}	16.7 ± 0.7^{b}
Mn (fg·μm^{-3})	0.09 ± 0.01^{a}	0.13 ± 0.03^{a}	0.12 ± 0.02^{a}
Fe (fg·μm^{-3})	0.39 ± 0.07^{a}	0.38 ± 0.04^{a}	0.34 ± 0.15^{a}
Cu (fg·μm^{-3})	0.011 ± 0.002^{a}	0.006 ± 0.001^{b}	0.003 ± 0.001^{b}
Zn (fg·μm^{-3})	0.025 ± 0.004^{a}	0.040 ± 0.007^{b}	0.043 ± 0.004^{b}
Br (fg·μm^{-3})	0.0005 ± 0.0001^{a}	0.0028 ± 0.0005^{b}	0.0027 ± 0.0001^{b}
Sr (fg·μm^{-3})	0.0025 ± 0.0007^{a}	0.1903 ± 0.0556^{b}	0.1437 ± 0.0059^{b}
Cd (fg·μm^{-3})	0^{a}	0.22 ± 0.05^{b}	0.12 ± 0.05^{b}
Pb (fg·μm^{-3})	0^{a}	0.00035 ± 0.00002^{b}	0.0005 ± 0.0004^{b}
Co (fg·μm^{-3})	0	0	0
Ni (fg·μm^{-3})	0^{a}	0.0041 ± 0.0009^{b}	0.0050 ± 0.0006^{b}
As (fg·μm^{-3})	0^{a}	0.0017 ± 0.0005^{b}	0.0014 ± 0.0001^{b}

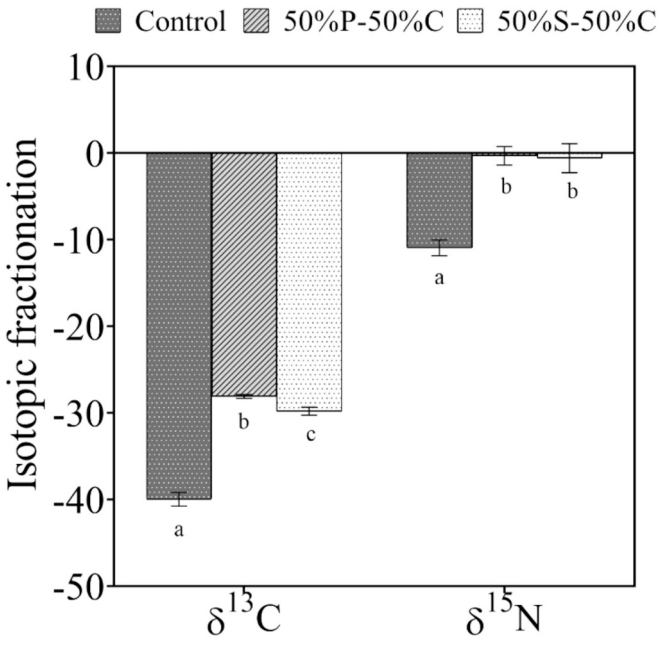

Fig. 6. C and N isotopic fractionation of the algal consortium acclimated to two different combinations of sewage streams compared to control conditions (50 % primary effluent (P)-50 % centrate (C); 50 % secondary effluent (*S*)-50%C). Experimental data are presented as mean ± SD. Letters represent significant differences among the conditions ($p < 0.05$).

control medium (Dawson et al., 2002; Frank and David Evans, 1997). However, the P content per cell volume was, on average, three times higher when the algae were grown in WW, changing the overall C:N:P ratio (Table 4). There are two primary explanations for this finding. As the achieved biomass was comparable among the growth conditions (Table 2), the sewage-acclimated algae were likely to show luxury uptake of P (Yao et al., 2011). This mechanism allows algae to take up and store higher quotas of P as polyphosphate than immediately needed, thereby ensuring a supply of P during long-term periods of P scarcity and/or environmental stress (Li et al., 2018; Solovchenko et al., 2019). In addition, extra P may have precipitated and adsorbed onto the cell walls or membranes of the microalgae. This phenomenon is consistent with the higher amounts of micronutrients (such as Ca and K) found in cells acclimated to sewage streams (Table 4) (further explained in Section 3.3). Although the cells were washed before analysis, some precipitated residuals could have remained in the algal biomass. Moreover, only a few elements were detected in WW-acclimated algae: Cd, Pb, As, and Ni (Table 4). These findings suggest that microalgae can remove certain toxic metals in trace amounts, as has been reported in previous studies (Leong and Chang, 2020). This would also imply that if microalgae biomass is exploited for biofertiliser production, the presence of metals should be considered, as the metal content in biofertilisers is generally limited by law. At the EU level, the maximum metal concentrations in the Product Function Categories (PFC) of fertilising products defined in EU Regulation 2019/1009 are listed in Table S6. Urban WW normally contains low amounts of metals; therefore, these limits should not be a significant barrier to the commercialisation of microalgae biofertilisers. Another challenge for the use of biomass as a fertiliser could be related to possible microbial contamination from both WW sources and external sources (Molina-Grima et al., 2022). This contamination can affect the long-term performance of the microalgae culture and the quality of the bio-products obtained from the microalgae biomass. Considering the use of microalgal biomass to produce biofertilizers and/or biostimulants (Álvarez-González et al., 2022; Hou et al., 2024), EU-Regulation 2019/1009 establishes a maximum *E. coli* concentration of 1000 cfu/g and states that *Salmonella* spp. must be absent in 25 g of the final bio-product. Consequently, downstream disinfection processes, such as composting, are needed to ensure that the biofertilisers/biostimulants produced are safe. However, further research is required to confirm this from the perspective of biomass commercialisation.

Overall, the algal growth results and biomass biochemical composition proved that the consortium efficiently acclimated to both sewage streams (primary and secondary effluents mixed with centrate). Therefore, from the microbiological point of view, the microalgae consortium tested could be integrated in both scenarios simulated in this study (Fig. S1). The acclimation phase of the algal consortium not only involves phenotypic changes in a single species but also the proportion among species, increasing the capacity of this biological system to overcome external disturbances, thus providing a strong and efficient tool for bioremediation. In this case, both shifts in species composition (i.e., towards smaller species) and metabolism (i.e., towards mixotrophy) occurred in the acclimated consortium which maintained biomass and macromolecular homeostasis per unit volume (Table 2). However, some reduction in the photosynthetic activity of WW-grown microalgal consortia was observed, implying certain challenges in upscaling the system. Maintaining high efficiency during outdoor microalgal cultivation is highly challenging, especially in the long term. Indeed, several authors have reported limited values of photosynthetic efficiencies in large-scale PBRs, accounting for approximately 1.5–2 % instead of the theoretical maximum values of 10–12 % (Nwoba et al., 2019; Raeisossadati et al., 2019). Because many factors can negatively affect microalgal photosynthetic activity, the fast adaptive response of microalgae to variations in this process is barely understood. This study provides a hypothesis on the possible acclimation responses of green microalgae cultivated in sewage and provides information that can be used in future research. Future studies should continuously monitor the photosynthetic efficiency of the microalgal culture to better control the operating parameters. Some authors have reported preliminary results on this (Masojídek et al., 2022; González-Camejo et al., 2020a; Resman et al., 2023). However, the early detection of the causes that reduce photosynthetic efficiency to foresee and mitigate them is highly challenging and still at a very early stage.

Table 5
Remediation capacity of the microalgae cultures cultivated in WW media. Letters indicate significant differences among the conditions ($p < 0.05$).

	Concentration at the end of tests		Removal efficiency	
	50%P-50%C	50%S-50%C	50%P-50%C	50%S-50%C
pH	10.9 ± 0.2[a]	10.8 ± 0.2[a]	–	–
Conductivity ($\mu S \cdot cm^{-1}$)	1208 ± 53[a]	1195 ± 71[a]	7 ± 2 %[a]	9 ± 5 %[a]
Alkalinity ($mgCaCO_3 \cdot L^{-1}$)	88 ± 14[a]	82 ± 17[a]	73 ± 12 %[a]	70 ± 15 %[a]
sCOD ($mgCOD \cdot L^{-1}$)	67 ± 3[a]	66 ± 11[a]	83 ± 1 %[a]	87 ± 2 %[a]
NH_4 ($mgN \cdot L^{-1}$)	0.9 ± 1.4[a]	0.1 ± 0.0[a]	98 ± 3 %[a]	99.80 %[a]
Cl ($mgCl \cdot L^{-1}$)	229 ± 4[a]	236 ± 6 [a]	–	–
NO_2 ($mgN \cdot L^{-1}$)	0.0 ± 0.0[a]	0.0 ± 0.0[a]	100.00 %[a]	100.00 %[a]
NO_3 ($mgN \cdot L^{-1}$)	0.9 ± 1.7[a]	0.1 ± 0.1[a]	91 ± 9 %[a]	98 ± 4 %[a]
DIN ($mgN \cdot L^{-1}$)	1.8 ± 1.9[a]	0.2 ± 0.1[a]	96.4 ± 3.6 %[a]	99.7 ± 0.2 %[a]
PO_4 ($mgP \cdot L^{-1}$)	0.01 ± 0.01[a]	0.01 ± 0.01[a]	99.8 ± 0.2 %[a]	99.8 ± 0.2 %[a]
SO_4 ($mgSO4 \cdot L^{-1}$)	52 ± 2[a]	47 ± 1[a]	–	–
Na ($mgNa \cdot L^{-1}$)	151 ± 6[a]	146 ± 4[a]	–	–
K ($mgK \cdot L^{-1}$)	26 ± 2[a]	26 ± 2[a]	19 ± 8 %[a]	23 ± 7 %[a]
Mg ($mgMg \cdot L^{-1}$)	17 ± 3[a]	18 ± 3[a]	49 ± 9 %[a]	47 ± 9 %[a]
Ca ($mgCa \cdot L^{-1}$)	86 ± 32[a]	77 ± 34[a]	32 ± 25 %[a]	30 ± 28 %[a]

3.3. WW remediation

Table 5 shows the removal capacity of the acclimated microalgal cultures after the entire duration of the test (9 days). Control values were not considered in these calculations because this medium contained nutrients under replete conditions to avoid nutrient limitation; therefore, their removal values would not be comparable to those obtained for WW. All tests showed proficient removal of N and P, being over 96 % for both mixtures of WW, with no significant differences between both treatment scenarios. This is probably related to the fact that primary and secondary effluents had similar compositions, with differences mainly in their nutrient concentrations (Mohsenpour et al., 2021). The results are promising as they showed that the consortium removed nutrients from both combinations of WW to values below the legal limits reported in the updated version of the Urban WW Treatment Directive (COM, 2024) of <10 mg N·L^{-1} and <0.7 mg P·L^{-1} for WWTPs between 10,000–150,000 p.e. These results were surprising because previous studies indicated that the proportion of centrate should not exceed 30 % because of its potential toxicity to microalgae (Clagnan et al., 2022; Romero-Villegas et al., 2018). However, these values were obtained for a cultivation time of nine days, which is too long for the system to be feasible on a large scale. According to Ruiz et al. (2013), the optimal HRT should be inversely related to the growth rate (HRT $= 2{\cdot}\mu^{-1}$); which in this study corresponds to 3–3.3 days (according to the growth rates shown in Table 2). Because continuous outdoor operation can significantly affect the performance of the microalgae consortium (González-Camejo et al., 2020b), the nutrient removal efficiencies obtained in this study cannot be directly extrapolated to large-scale photobioreactors. Thus, it will be necessary to confirm whether the microalgae consortium would be able to operate at this HRT under outdoor conditions in the long term to achieve appropriate water polishing or whether the influent loading rate should be decreased during continuous operation.

In terms of carbon removal, COD removal efficiencies were significantly lower than those of the nutrients, accounting for 83.0 ± 0.9 % and 87.4 ± 2.2 % for 50%P-50%C and 50%S-50%C, respectively, probably because of the EPS that were released to the water media, especially when microalgae grew in secondary effluent rather than primary (Fig. S5). As EPS are normally released in higher amounts when microalgae are under stressful conditions (Belachqer-El Attar et al., 2023; Novoa et al., 2020), WW containing secondary effluent might present some physicochemical characteristics that stress microalgae to a higher degree than primary effluent. This can be relevant for the upscaling of the microalgae system because EPS, which are organic compounds, can decrease the quality of treated water and hinder the separation of microalgae biomass from water if filtration systems are used to do so (González-Camejo et al., 2020c). In contrast, EPS can improve biomass separation if other harvesting systems, such as sedimentation or air flotation, are used (de Morais et al., 2023; Sun et al., 2023). However, despite this EPS production, COD at the end of the tests were in all cases under the legal concentration limit of 125 mgCOD·L^{-1} (COM, 2024), as displayed in Table 5. Some C removal may have been due to the activity of the bacteria present in the WW. However, as cultivation conditions were set to favour microalgal growth, the activity of bacteria was expected to be less relevant than that of microalgae, as observed in previous studies (González-Camejo et al., 2020a). Some removal of organic carbon could have also been due to the mixotrophic activity of microalgae, as suggested by the higher fractionation of $\delta^{13}C$ in both WW media in comparison to control conditions (Fig. 6).

It is important to note the high pH-values obtained for both 50%P-50%C and 50%S-50%C media, which accounted for 10.9 ± 0.2 and 10.8 ± 0.2, respectively, at the end of the tests. This was due to microalgal photosynthetic activity, which increases pH, and the fact that alkalinity is not high enough to buffer the pH. These high pH values have several negative effects. First, this treated water is not suitable for reuse in agriculture, as the pH must be within the range of 6–9.5 (DM 185-2003). In addition, these high values favour the acid-basic equilibrium of ammonia (González-Camejo et al., 2020b), which is toxic to microalgae and can volatilise. As tests were carried out in 500-mL Erlenmeyer flasks, NH_3 losses were assumed to be minimal, but they could be highly relevant if upscaled in open mixed photobioreactors (Mantovani et al., 2020). Moreover, pH values >9 are often responsible for increasing the amount of precipitates in the mixed liquor (Iasimone et al., 2018). In the current study, solubility equilibria were calculated for 50%P-50%C and 50%S-50%C under the most unfavourable conditions, obtaining positive values in the saturation indices (indicating oversaturation) for the same compounds in both WW combinations (Table S8). According to these results, calcium, carbonate, magnesium, and phosphate ions should have mainly been in precipitated form (Table S9); therefore, some of these ions were not bioavailable and were probably adsorbed on the cell surface instead of being acquired and assimilated. This also explains the removal of Ca and Mg from both WW, whereas the remaining ions remained approximately constant. Consequently, low-conductivity removal was observed in both WW media (Table 5). Other authors have reported significant removal of ions by microalgae cells. For instance, Znad et al. (2018) reported Ca and Mg removal of 100 % and 82 %, respectively, by microalgae cultivated in primary effluents and Ca and Mg removal of 66 % and 63 %, respectively, in the case of secondary effluents. However, it is unclear whether these ions were assimilated by microalgae or precipitated, as the pH values were over 8.5 and no further information in this respect was provided. This information is highly relevant for upscaling the process as it highlights the necessity of controlling the pH of the cultivation system to avoid all the aforementioned issues. Several options have been reported to control pH by providing CO_2 to the culture medium via raw flue gas, commercial purified CO_2, and the addition of reagents containing carbonates or bicarbonates, but they imply significant operating costs that vary between 10 and 380 USD·tCO_2^{-1} (Zheng et al., 2018).

3.4. Economic evaluation

As mentioned above, WW containing both primary and secondary effluents showed similar growth rates, biomass characteristics, and bioremediation capacities. However, the higher amount of EPS in the medium with secondary effluent suggests that the microalgae suffered from more significant stress than in the medium with primary effluent. Additionally, from an operational point of view, using microalgae to treat primary effluent implies a reduction in the amount of WW treated by the activated sludge system, unlike the use of secondary effluent. In the latter case, a step for COD removal from the activated sludge system was required (Fig. S1). Hence, upscaling of this technology was performed considering only the primary effluent mixed with centrate

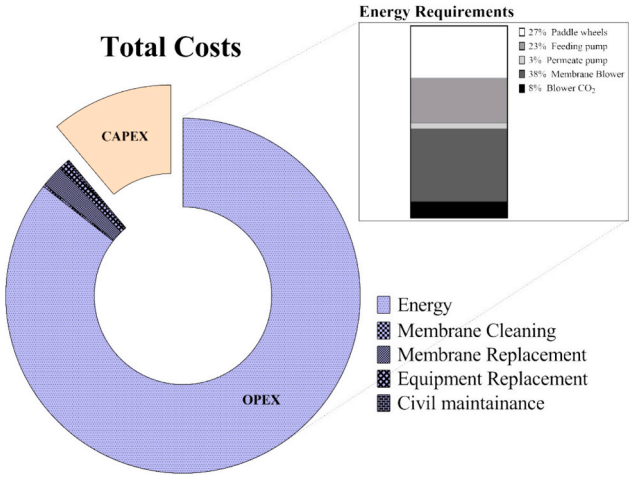

Fig. 7. Results of the economic assessment of the microalgae cultivation system.

(Fig. 1).

The economic assessment shows that building and operating a microalgae cultivation system to be integrated with urban WWTP streams would imply a total cost of 0.109 €·m^{-3} water treated (only considering the microalgae cultivation and harvesting system as shown in Fig. 7). This number is promising as conventional treatment processes based on activated sludge can present costs of up to 0.22 €·m^{-3} (Acien et al., 2023). This suggests that integrating a microalgal cultivation system could save money in WW treatment operations. Of the total costs, 89 % corresponded to OPEX and only 11 % to CAPEX. Within OPEX, energy costs were the dominant contributor, at 86 % (Fig. 7). However, the total energy consumption, i.e., 0.377 Kwh·m^{-3}, was significantly lower than the typical energy consumption of activated sludge systems of 0.5–0.8 Kwh·m^{-3} (Acien et al., 2023). The main contributor to energy consumption was the harvesting system as membrane scouring and feeding and permeate pumps accounted for 0.243 Kwh·m^{-3}, equivalent to 65 % of the total energy consumption (Fig. 7). This is promising as the energy needed for harvesting microalgae using membranes has been reported to be in the range of 0.17–8 kWh·m^{-3} (Mora-Sánchez et al., 2024; Zhao et al., 2023).

The cost calculated here can be reduced. For instance, if the microalgae biomass produced would be sold as regular fertiliser (0.1 €·kg^{-1} with 100 % conversion, according to Acien et al., 2023), the OPEX could be reduced, but only by 0.001 €·m^{-3}. It would be thus more impactful to sell the biomass as organic fertiliser (0.5 €·kg^{-1}) or biostimulant (1 €·kg^{-1}) to make the system more profitable. However, the production of biostimulants implies higher technical complexity in obtaining the appropriate peptides which can bio-stimulate plants (Amaya-Santos et al., 2022), whereas the production and commercialisation of organic fertilisers can present certain difficulties in terms of permits and legal issues. Further research is thus needed to evaluate the most feasible option for recovering microalgal biomass to maximise the benefits obtained without significantly increasing the complexity of the facility. Another way to reduce the total cost is to obtain subsidies from public and/or private funding that supports environmentally friendly technologies (Cipolletta et al., 2021). For instance, in Italy, the National Recovery and Resilience Plan (Piano Nazionale di Ripresa e Resilienza; PNRR) has provided 600 million to adapt the Italian water sector system to the provisions of the updated European Directives related to water (PNRR, 2022).

4. Conclusions

Based on the experimental results, relevant information for upscaling the microalgal cultivation process was obtained. The main conclusions were as follows:

i) The tested microalgal consortium could be a valuable, robust, and flexible biosystem to be integrated with sewage treatment facilities as they successfully acclimated to WW by modulating species composition and metabolism and efficiently bioremediated primary and secondary effluents mixed with up to 50 % of centrate.
ii) No major differences were observed in biochemical composition, biomass yield, and bioremediation capacity between the two mixed sewage streams (primary and secondary effluents mixed with centrate). However, microalgae grown in the secondary effluent released higher amounts of EPS.
iii) The optimal HRT would correspond to 3.3 days but outdoor operation would be expected to be adjusted to cope with the limitation in photosynthetic efficiency that was determined using fluorescence-derived parameters.
iv) Several factors may have contributed to the reduction in photosynthetic activity, mainly changes in species composition, photoinhibition, and variation in metabolic activity inducing mixotrophic metabolism.
v) CO_2 addition is recommended for upscaling the system to avoid excessive pH values and significant nutrient loss.
vi) Preliminary economic assessment reported the cost of the microalgae system to be 0.109 €·m^{-3} water treated, significantly lower than conventional treatment processes.

CRediT authorship contribution statement

M.G. Chieti: Formal analysis, Data curation. **A. Petrucciani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **L. Mollo:** Validation, Methodology, Formal analysis, Data curation. **C. Gerotto:** Writing – review & editing, Supervision, Methodology. **A.L. Eusebi:** Supervision, Resources, Project administration. **F. Fatone:** Supervision, Resources, Project administration, Funding acquisition. **A. Norici:** Writing – review & editing, Supervision, Resources, Methodology. **J. González-Camejo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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